

*Application
for
United States Letters Patent*

To all whom it may concern:

Be it known that **Andrew R. Marks et al.**

have invented certain new and useful improvements in

P27 PREVENTS CELLULAR MIGRATION

of which the following is a full, clear and exact description.

P27 PREVENTS CELLULAR MIGRATION

5 The invention disclosed herein was made with
Government support under grant numbers RO1HL56180,
RO1A139794, and RO3TW00949 from the National
Institutes of Health, U.S. Department of Health and
Human Services. Accordingly, the U.S. Government has
10 certain rights in this invention.

Background Of The Invention

15 Throughout this application, various publications are
referenced in parentheses by author and year. Full
citations for these references may be found at the
end of the specification immediately preceding the
claims. The disclosures of these publications in
their entireties are hereby incorporated by reference
20 into this application to more fully describe the
state of the art to which this invention pertains.

25 Vascular smooth muscle cell (SMC) migration is
believed to play a major role in the pathogenesis of
many vascular diseases, such as atherosclerosis and
restenosis after both percutaneous transluminal
angioplasty (PTCA) and coronary stenting (Schwartz,
1997). In normal blood vessels, the majority of SMC
reside in the media or middle coat of the vessel,
30 where they are quiescent and possess a "contractile"
phenotype, characterized by the abundance of actin-
and myosin-containing filaments. In disease states,
SMCs migrate from the media to the intima or inner

coat of the blood vessel. The process of SMC migration in pathological states involves the synthesis of extracellular matrix, protease enzymes, growth factors such as platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF), and cytokines that further contribute to proliferation and migration (Clowes and Schwartz, 1985; Ferns et al., 1991; Grotendorst et al., 1981; Ihnatowycz et al., 1981; Jawien et al., 1992). Fibroblast growth factor-2 (FGF-2) appears to modulate SMC migration by changing extracellular matrix (ECM)- β 1 integrin interactions (Pickering et al., 1997). FGF-2 augments SMC surface expression of α 2 β 1, α 3 β 1 and α v β 1 integrins, thereby resulting in enhanced cellular motility through disassembly of the α -actin stress fiber network (Pickering et al., 1997).

Rapamycin, a macrolide antibiotic, inhibits SMC proliferation both *in vitro* and *in vivo* by blocking cell cycle progression at the transition between the first gap (G1) and DNA synthesis (S) phases (Cao et al., 1995; Gallo et al., 1999; Gregory et al., 1993; Marx et al., 1995). The inhibition of cellular proliferation is associated with a marked reduction in cell cycle dependent kinase activity and in retinoblastoma protein phosphorylation *in vitro* (Marx et al., 1995) and *in vivo* (Gallo et al., 1999). Down-regulation of the cyclin-dependent kinase inhibitor (CDKI) p27^{kip1} by mitogens is blocked by rapamycin (Kato et al., 1994; Nourse et al., 1994). Pre-treatment of rat and human SMC with rapamycin (2 nM) for 48 hours inhibited PDGF-induced SMC migration in a modified Boyden chamber. However, acute rapamycin

treatment (6 hours) of rat and human SMC had no effect on migration, suggesting that longer exposure to rapamycin is essential for its anti-migratory actions. In support of these findings, acute 6 hour treatment with rapamycin (1-100 nM), wortmannin and LY294002 of both SMC and Swiss 3T3 cells failed to inhibit PDGF-induced chemotaxis (Higaki et al., 1996). The findings that rapamycin possesses both anti-proliferative and anti-migratory SMC properties led to the suggestion that rapamycin may have important applications in the treatment of disorders such as accelerated arteriopathy that occurs in transplanted hearts and restenosis after percutaneous transluminal angioplasty and placement of coronary stents (Marx et al., 1995; Marx and Marks, 1999; Poon et al., 1996). Rapamycin significantly inhibited neointimal proliferation in a porcine angioplasty model (Gallo et al., 1999) and reversed chronic graft vascular disease in a rodent heart allograft model (Poston et al., 1999). Recent clinical studies have implicated the importance of rapamycin in treating stent restenosis (Sousa et al., 2000).

In $p27^{kip1}$ (-/-) knockout mice, relative rapamycin resistance was demonstrated, and in rapamycin resistant myogenic cells, constitutively low levels of $p27^{kip1}$ were observed, which were not increased with serum withdrawal and rapamycin (Luo et al., 1996). These findings suggested that the ability to block $p27^{kip1}$ down-regulation contributes to the growth inhibitory effects of rapamycin. Transfection of the cyclin-dependent kinase inhibitor $p21^{cip1}$ was shown to inhibit the spreading and attachment of SMC to

5

10

15

Summary Of The Invention

5 This invention is directed to a method of preventing migration of a cell by increasing intracellular cyclin-dependent kinase inhibitor p27 activity.

10 The invention provides a method of treating a subject's cardiovascular disease, which comprises administering to the subject a compound which increases intracellular cyclin-dependent kinase inhibitor p27 activity, thereby alleviating the subject's cardiovascular disease.

15 The invention provides a method of inhibiting tumor metastasis in a subject, which comprises administering to the subject a compound which increases intracellular cyclin-dependent kinase inhibitor p27 activity, thereby inhibiting tumor metastasis.

20 The invention provides a method of identifying a chemical compound that inhibits cellular migration, which comprises contacting cells whose migration is inhibited when intracellular cyclin-dependent kinase inhibitor p27 activity is increased, or contacting an extract from said cells, with the chemical compound under conditions suitable for increasing p27 activity, and detecting an increase in p27 activity in the presence of the chemical compound so as to
25
30 thereby identify the chemical compound as a compound which inhibits cellular migration.

The invention provides a method of screening a plurality of chemical compounds not known to inhibit cellular migration to identify a chemical compound which inhibits cellular migration, which comprises:

- 5 (a) contacting cells whose migration is inhibited when intracellular cyclin-dependent kinase inhibitor p27 activity is increased, or contacting an extract from said cells, with the plurality of chemical compounds under conditions
10 suitable for increasing p27 activity;
- (b) determining if p27 activity is increased in the presence of the plurality of chemical compounds; and if so
- (c) separately determining if p27 activity is
15 increased in the presence of each compound included in the plurality of chemical compounds, so as to thereby identify any compound included therein as a compound which inhibits cellular migration.

20

The invention provides a chemical compound identified by any of the methods described herein.

- The invention provides a pharmaceutical composition
- 25 comprising (a) an amount of a chemical compound identified using any of the methods described herein, or a novel structural and functional homolog or analog thereof, capable of passing through a cell membrane and effective to increase intracellular
30 cyclin-dependent kinase inhibitor p27 activity and
 - (b) a pharmaceutically acceptable carrier capable of passing through the cell membrane.

The invention provides a pharmaceutical composition comprising an amount of a chemical compound identified using any of the methods described herein effective to inhibit cellular migration and a
5 pharmaceutically acceptable carrier.

The invention provides a method for preparing a composition which comprises admixing a carrier and a pharmaceutically effective amount of a chemical
10 compound identified by any of the methods described herein or a novel structural and functional analog or homolog thereof.

The invention provides a method for making a composition of matter which inhibits cellular migration which comprises identifying a chemical
15 compound using any of the methods described herein, and then synthesizing the chemical compound or a novel structural and functional analog or homolog
20 thereof.

The invention provides a method of treating a subject with a cardiovascular disease which comprises administering to the subject a therapeutically
25 effective amount of a chemical compound identified by any of the methods described herein, or a novel structural and functional analog or homolog thereof.

The invention provides a method of inhibiting tumor metastasis in a subject which comprises administering
30 to the subject a therapeutically effective amount of a chemical compound identified by any of the methods

described herein, or a novel structural and functional analog or homolog thereof.

5 The invention provides a use of a chemical compound identified by any of the methods described herein for the preparation of a pharmaceutical composition for treating an abnormality, wherein the abnormality is alleviated by inhibiting cellular migration.

10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
66
67
68
69
70
71
72
73
74
75
76
77
78
79
80
81
82
83
84
85
86
87
88
89
90
91
92
93
94
95
96
97
98
99
100
101
102
103
104
105
106
107
108
109
110
111
112
113
114
115
116
117
118
119
120
121
122
123
124
125
126
127
128
129
130
131
132
133
134
135
136
137
138
139
140
141
142
143
144
145
146
147
148
149
150
151
152
153
154
155
156
157
158
159
160
161
162
163
164
165
166
167
168
169
170
171
172
173
174
175
176
177
178
179
180
181
182
183
184
185
186
187
188
189
190
191
192
193
194
195
196
197
198
199
200
201
202
203
204
205
206
207
208
209
210
211
212
213
214
215
216
217
218
219
220
221
222
223
224
225
226
227
228
229
230
231
232
233
234
235
236
237
238
239
240
241
242
243
244
245
246
247
248
249
250
251
252
253
254
255
256
257
258
259
260
261
262
263
264
265
266
267
268
269
270
271
272
273
274
275
276
277
278
279
280
281
282
283
284
285
286
287
288
289
290
291
292
293
294
295
296
297
298
299
300
301
302
303
304
305
306
307
308
309
310
311
312
313
314
315
316
317
318
319
320
321
322
323
324
325
326
327
328
329
330
331
332
333
334
335
336
337
338
339
340
341
342
343
344
345
346
347
348
349
350
351
352
353
354
355
356
357
358
359
360
361
362
363
364
365
366
367
368
369
370
371
372
373
374
375
376
377
378
379
380
381
382
383
384
385
386
387
388
389
390
391
392
393
394
395
396
397
398
399
400
401
402
403
404
405
406
407
408
409
410
411
412
413
414
415
416
417
418
419
420
421
422
423
424
425
426
427
428
429
430
431
432
433
434
435
436
437
438
439
440
441
442
443
444
445
446
447
448
449
450
451
452
453
454
455
456
457
458
459
460
461
462
463
464
465
466
467
468
469
470
471
472
473
474
475
476
477
478
479
480
481
482
483
484
485
486
487
488
489
490
491
492
493
494
495
496
497
498
499
500
501
502
503
504
505
506
507
508
509
510
511
512
513
514
515
516
517
518
519
520
521
522
523
524
525
526
527
528
529
530
531
532
533
534
535
536
537
538
539
540
541
542
543
544
545
546
547
548
549
550
551
552
553
554
555
556
557
558
559
560
561
562
563
564
565
566
567
568
569
570
571
572
573
574
575
576
577
578
579
580
581
582
583
584
585
586
587
588
589
590
591
592
593
594
595
596
597
598
599
600
601
602
603
604
605
606
607
608
609
610
611
612
613
614
615
616
617
618
619
620
621
622
623
624
625
626
627
628
629
630
631
632
633
634
635
636
637
638
639
640
641
642
643
644
645
646
647
648
649
650
651
652
653
654
655
656
657
658
659
660
661
662
663
664
665
666
667
668
669
670
671
672
673
674
675
676
677
678
679
680
681
682
683
684
685
686
687
688
689
690
691
692
693
694
695
696
697
698
699
700
701
702
703
704
705
706
707
708
709
710
711
712
713
714
715
716
717
718
719
720
721
722
723
724
725
726
727
728
729
730
731
732
733
734
735
736
737
738
739
740
741
742
743
744
745
746
747
748
749
750
751
752
753
754
755
756
757
758
759
760
761
762
763
764
765
766
767
768
769
770
771
772
773
774
775
776
777
778
779
780
781
782
783
784
785
786
787
788
789
790
791
792
793
794
795
796
797
798
799
800
801
802
803
804
805
806
807
808
809
810
811
812
813
814
815
816
817
818
819
820
821
822
823
824
825
826
827
828
829
830
831
832
833
834
835
836
837
838
839
840
841
842
843
844
845
846
847
848
849
850
851
852
853
854
855
856
857
858
859
860
861
862
863
864
865
866
867
868
869
870
871
872
873
874
875
876
877
878
879
880
881
882
883
884
885
886
887
888
889
890
891
892
893
894
895
896
897
898
899
900
901
902
903
904
905
906
907
908
909
910
911
912
913
914
915
916
917
918
919
920
921
922
923
924
925
926
927
928
929
930
931
932
933
934
935
936
937
938
939
940
941
942
943
944
945
946
947
948
949
950
951
952
953
954
955
956
957
958
959
960
961
962
963
964
965
966
967
968
969
970
971
972
973
974
975
976
977
978
979
980
981
982
983
984
985
986
987
988
989
990
991
992
993
994
995
996
997
998
999
1000
1001
1002
1003
1004
1005
1006
1007
1008
1009
1010
1011
1012
1013
1014
1015
1016
1017
1018
1019
1020
1021
1022
1023
1024
1025
1026
1027
1028
1029
1030
1031
1032
1033
1034
1035
1036
1037
1038
1039
1040
1041
1042
1043
1044
1045
1046
1047
1048
1049
1050
1051
1052
1053
1054
1055
1056
1057
1058
1059
1060
1061
1062
1063
1064
1065
1066
1067
1068
1069
1070
1071
1072
1073
1074
1075
1076
1077
1078
1079
1080
1081
1082
1083
1084
1085
1086
1087
1088
1089
1090
1091
1092
1093
1094
1095
1096
1097
1098
1099
1100
1101
1102
1103
1104
1105
1106
1107
1108
1109
1110
1111
1112
1113
1114
1115
1116
1117
1118
1119
1120
1121
1122
1123
1124
1125
1126
1127
1128
1129
1130
1131
1132
1133
1134
1135
1136
1137
1138
1139
1140
1141
1142
1143
1144
1145
1146
1147
1148
1149
1150
1151
1152
1153
1154
1155
1156
1157
1158
1159
1160
1161
1162
1163
1164
1165
1166
1167
1168
1169
1170
1171
1172
1173
1174
1175
1176
1177
1178
1179
1180
1181
1182
1183
1184
1185
1186
1187
1188
1189
1190
1191
1192
1193
1194
1195
1196
1197
1198
1199
1200
1201
1202
1203
1204
1205
1206
1207
1208
1209
1210
1211
1212
1213
1214
1215
1216
1217
1218
1219
1220
1221
1222
1223
1224
1225
1226
1227
1228
1229
1230
1231
1232
1233
1234
1235
1236
1237
1238
1239
1240
1241
1242
1243
1244
1245
1246
1247
1248
1249
1250
1251
1252
1253
1254
1255
1256
1257
1258
1259
1260
1261
1262
1263
1264
1265
1266
1267
1268
1269
1270
1271
1272
1273
1274
1275
1276
1277
1278
1279
1280
1281
1282
1283
1284
1285
1286
1287
1288
1289
1290
1291
1292
1293
1294
1295
1296
1297
1298
1299
1300
1301
1302
1303
1304
1305
1306
1307
1308
1309
1310
1311
1312
1313
1314
1315
1316
1317
1318
1319
1320
1321
1322
1323
1324
1325
1326
1327
1328
1329
1330
1331
1332
1333
1334
1335
1336
1337
1338
1339
1340
1341
1342
1343
1344
1345
1346
1347
1348
1349
1350
1351
1352
1353
1354
1355
1356
1357
1358
1359
1360
1361
1362
1363
1364
1365
1366
1367
1368
1369
1370
1371
1372
1373
1374
1375
1376
1377
1378
1379
1380
1381
1382
1383
1384
1385
1386
1387
1388
1389
1390
1391
1392
1393
1394
1395
1396
1397
1398
1399
1400
1401
1402
1403
1404
1405
1406
1407
1408
1409
1410
1411
1412
1413
1414
1415
1416
1417
1418
1419
1420
1421
1422
1423
1424
1425
1426
1427
1428
1429
1430
1431
1432
1433
1434
1435
1436
1437
1438
1439
1440
1441
1442
1443
1444
1445
1446
1447
1448
1449
1450
1451
1452
1453
1454
1455
1456
1457
1458
1459
1460
1461
1462
1463
1464
1465
1466
1467
1468
1469
1470
1471
1472
1473
1474
1475
1476
1477
1478
1479
1480
1481
1482
1483
1484
1485
1486
1487
1488
1489
1490
1491
1492
1493
1494
1495
1496
1497
1498
1499
1500
1501
1502
1503
1504
1505
1506
1507
1508
1509
1510
1511
1512
1513
1514
1515
1516
1517
1518
1519
1520
1521
1522
1523
1524
1525
1526
1527
1528
1529
1530
1531
1532
1533
1534
1535
1536
1537
1538
1539
1540
1541
1542
1543
1544
1545
1546
1547
1548
1549
1550
1551
1552
1553
1554
1555
1556
1557
1558
1559
1560
1561
1562
1563
1564
1565
1566
1567
1568
1569
1570
1571
1572
1573
1574
1575
1576
1577
1578
1579
1580
1581
1582
1583
1584
1585
1586
1587
1588
1589
1590
1591
1592
1593
1594
1595
1596
1597
1598
1599
1600
1601
1602
1603
1604
1605
1606
1607
1608
1609
1610
1611
1612
1613
1614
1615
1616
1617
1618
1619
1620
1621
1622
1623
1624
1625
1626
1627
1628
1629
1630
1631
1632
1633
1634
1635
1636
1637
1638
1639
1640
1641
1642
1643
1644
1645
1646
1647
1648
1649
1650
1651
1652
1653
1654
1655
1656
1657
1658
1659
1660
1661
1662
1663
1664
1665
1666
1667
1668
1669
1670
1671
1672
1673
1674
1675
1676
1677
1678
1679
1680
1681
1682
1683
1684
1685
1686
1687
1688
1689
1690
1691
1692
1693
1694
1695
1696
1697
1698
1699
1700
1701
1702
1703
1704
1705
1706
1707
1708
1709
1710
1711
1712
1713
1714
1715
1716
1717
1718
1719
1720
1721
1722
1723
1724
1725
1726
1727
1728
1729
1730
1731
1732
1733
1734
1735
1736
1737
1738
1739
1740
1741
1742
1743
1744
1745
1746
1747
1748
1749
1750
1751
1752
1753
1754
1755
1756
1757
1758
1759
1760
1761
1762
1763
1764
1765
1766
1767
1768
1769
1770
1771
1772
1773
1774
1775
1776
1777
1778
1779
1780
1781
1782
1783
1784
1785
1786
1787
1788
1789
1790
1791
1792
1793
1794
1795
1796
1797
1798
1799
1800
1801
1802
1803
1804
1805
1806
1807
1808
1809
1810
1811
1812
1813
1814
1815
1816
1817
1818
1819
1820
1821
1822
1823
1824
1825
1826
1827
1828
1829
1830
1831
1832
1833
1834
1835
1836
1837
1838
1839
1840
1841
1842
1843
1844
1845
1846
1847
1848
1849
1850
1851
1852
1853
1854
1855
1856
1857
1858
1859
1860
1861
1862
1863
1864
1865
1866
1867
1868
1869
1870
1871
1872
1873
1874
1875
1876
1877
1878
1879
1880
1881
1882
1883
1884
1885
1886
1887
1888
1889
1890
1891
1892
1893
1894
1895
1896
1897
1898
1899
1900
1901
1902
1903
1904
1905
1906
1907
1908
1909
1910
1911
1912
1913
1914
1915
1916
1917
1918
1919
1920
1921
1922
1923
1924
1925
1926
1927
1928
1929
1930
1931
1932
1933
1934
1935
1936
1937
1938
1939
1940
1941
1942
1943
1944
1945
1946
1947
1948
1949
1950
1951
1952
1953
1954
1955
1956
1957
1958
1959
1960
1961
1962
1963
1964
1965
1966
1967
1968
1969
1970
1971
1972
1973
1974
1975
1976
1977
1978
1979
1980
1981
1982
1983
1984
1985
1986
1987
1988
1989
1990
1991
1992
1993
1994
1995
1996
1997
1998
1999
2000
2001
2002
2003
2004
2005
2006
2007
2008
2009
2010
2011
2012
2013
2014
2015
2016
2017
2018
2019
2020
2021
2022
2023
2024
2025
2026
2027
2028
2029
2030
2031
2032
2033
2034
2035
2036
2037
2038
2039
2040
2041
2042
2043
2044
2045
2046
2047
2048
2049
2050
2051
2052
2053
2054
2055
2056
2057
2058
2059
2060
2061
2062
2063
2064
2065
2066
2067
2068
2069
2070
2071
2072
2073
2074
2075
2076
2077
2078
2079
2080
2081
2082
2083
2084
2085
2086
2087
2088
2089
2090
2091
2092
2093
2094
2095
2096
2097
2098
2099
2100
2101
2102
2103
2104
2105
2106
2107
2108
2109
2110
2111
2112
2113
2114
2115
2116
2117
2118
2119
2120
2121
2122
2123
2124
2125
2126
2127
2128
2129
2130
2131
2132
2133
2134
2135
2136
2137
2138
2139
2140
2141
2142
2143
2144
2145
2146
2147
2148
2149
2150
2151
2152
2153
2154
2155
2156
2157
2158
2159
2160
2161
2162
2163
2164
2165
2166
2167
2168
2169
2170
2171
2172
2173
2174
2175
2176
2177
2178
2179
2180
2181
2182
2183
2184
2185
2186
2187
2188
2189
2190
2191
2192
2193
2194
2195
2196
2197
2198
2199
2200
2201
2202
2203
2204
2205
2206
2207
2208
2209
2210
2211
2212
2213
2214
2215
2216
2217
2218
2219
2220
2221
2222
2223

Brief Description Of The Figures

Figure 1A-D. Rapamycin potently inhibits migration in smooth muscle cells from wild type, but not p27 (-/-) knockout mice.

(A) Migration of SMCs isolated from wild type mice was determined in the modified Boyden chamber following rapamycin and FK506 treatment. Rapamycin (open bars; 1, 10 and 100 nM) significantly inhibited SMC migration, whereas FK506 demonstrated no effect (blackened bars). * $p < 0.05$ as compared to control. The inset shows an immunoblot demonstrating increased p27^{kip1} levels after rapamycin (100 nM for 48 hours) treatment (lane 2) as compared to untreated proliferating SMC (lane 1).

(B) Migration of SMCs isolated from p27(-/-) knockout mice was determined in the modified Boyden chamber following rapamycin and FK506 treatment. Only at high concentrations did rapamycin (open bars; 100 and 1000 nM) significantly inhibit SMC migration, whereas FK506 demonstrated no effect (blackened bars). * $p < 0.05$ as compared to control. The inset shows an immunoblot demonstrating the absence of p27^{kip1}.

(C and D) FK506 competes with rapamycin for binding to FKBP12 and inhibits the effects of rapamycin on wild type (C) and p27 (-/-) (D) SMC migration.

Figure 2A-B. Lack of effect of rapamycin on murine SMC adhesion.

5 Wild type (open bars) and p27(-/-) (blackened bars)
SMC were incubated with rapamycin for 48 hours before
plating onto either fibronectin (A) or laminin (B)
coated plates for 3 hours. The number of adhering
cells was determined with a Coulter counter in
triplicate and normalized to the number of untreated
10 wild type cells. No significant differences were
noted between treated and untreated cells.

Figure 3A-C. In vivo administration of rapamycin
15 potentially inhibits explant migration of SMC from wild
type but not p27(-/-) knockout animals.

(A) p27 (+/+), p27 (+/-) and p27 (-/-) mice were
injected with rapamycin (4 mg/kg/day) for 5 days.
The aortas were explanted, and migration of SMC was
20 quantified and is presented as the rapamycin-mediated
inhibition of migration as a % of control. Rapamycin
significantly inhibited migration in both p27 (+/+) and
p27 (+/-) SMC; rapamycin had no effect on p27 (-
/-) SMC explant migration

25 (B) p27 (+/+), p27 (+/-) and p27 (-/-) mice were
injected with rapamycin (9 mg/kg/day) for 7 days.
Rapamycin inhibited migration in p27 (+/+), p27 (+/-)
and p27 (-/-) SMC explants.

30 (C) p27 (+/+) and p27 (-/-) mice were injected with
taxol (20 mg/kg/day) for 7 days. Taxol inhibited
migration in p27 (+/+) and p27 (-/-) SMC.

Figure 4. Impaired migration-inhibitory response to C3 exoenzyme in SMC derived from p27 (-/-) knockout mice.

5

Migration of SMC isolated from wild type mice (open bars) and p27 (-/-) mice (blackened bars) was determined in the modified Boyden chamber following C3 exoenzyme (2 and 20 µg/ml) treatment for 16 hours. SMC derived from p27 (-/-) mice demonstrated a 25% relative migratory resistance to C3 exoenzyme.

10

* $p < 0.05$ as compared to control.

Figure 5. Rapamycin and C3 exoenzyme inhibit SMC migration through p27^{k1p1}-dependent and -independent pathways.

15

Rapamycin (Rapa)-FKBP12 inhibits target-of-rapamycin (TOR)-mediated activation/phosphorylation of protein translation modulators 4E-BP1 (a translation initiation factor) and p70 S6 kinase (S6 is a ribosomal protein) (Marx and Marks, 1999) and prevents mitogen-induced down-regulation of p27^{k1p1} through an unknown mechanism (dashed lines). Rapamycin inhibits SMC migration through p27^{k1p1}-dependent and -independent mechanisms. C3 exoenzyme, which specifically ADP ribosylates and inhibits RhoA, inhibits SMC migration through p27^{k1p1}-dependent and -independent (cytoskeleton changes) pathways.

20

25

30

bioRxiv preprint doi: <https://doi.org/10.1101/000000>; this version posted March 1, 2014. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Detailed Description Of The Invention

The present invention is directed to a method of preventing migration of a cell by increasing intracellular cyclin-dependent kinase inhibitor p27 activity. In different embodiments of the method, the cell is a smooth muscle cell or a tumor cell.

The invention provides a method of treating a subject's cardiovascular disease, which comprises administering to the subject a compound which increases intracellular cyclin-dependent kinase inhibitor p27 activity, thereby alleviating the subject's cardiovascular disease. In different embodiments, the cardiovascular disease is atherosclerosis, arteriopathy after heart transplantation, or restenosis after angioplasty or coronary stent placement.

The invention provides a method of inhibiting tumor metastasis in a subject, which comprises administering to the subject a compound which increases intracellular cyclin-dependent kinase inhibitor p27 activity, thereby inhibiting tumor metastasis.

In one embodiment of the methods described herein, cyclin-dependent kinase inhibitor p27 activity is increased by increasing C3 exoenzyme activity.

In different embodiments, cyclin-dependent kinase inhibitor p27 activity is increased by pharmacological techniques, by recombinant

techniques, or by gene therapy. Pharmacological techniques, recombinant techniques, and gene therapy techniques are well known in the art.

5 The invention provides a method of identifying a chemical compound that inhibits cellular migration, which comprises contacting cells whose migration is inhibited when intracellular cyclin-dependent kinase inhibitor p27 activity is increased, or contacting an
10 extract from said cells, with the chemical compound under conditions suitable for increasing p27 activity, and detecting an increase in p27 activity in the presence of the chemical compound so as to thereby identify the chemical compound as a compound
15 which inhibits cellular migration. In one embodiment, the chemical compound is not previously known to inhibit cellular migration.

20 The invention provides a method of screening a plurality of chemical compounds not known to inhibit cellular migration to identify a chemical compound which inhibits cellular migration, which comprises:

- 25 (a) contacting cells whose migration is inhibited when intracellular cyclin-dependent kinase inhibitor p27 activity is increased, or contacting an extract from said cells, with the plurality of chemical compounds under conditions suitable for increasing p27 activity;
- 30 (b) determining if p27 activity is increased in the presence of the plurality of chemical compounds; and if so
- (c) separately determining if p27 activity is increased in the presence of each compound

included in the plurality of chemical compounds,
so as to thereby identify any compound included
therein as a compound which inhibits cellular
migration.

5

In different embodiments of the methods described
herein, cyclin-dependent kinase inhibitor p27
activity is detected using immunoblots.

10 In different embodiments of the methods described
herein, the cells are smooth muscle cells or tumor
cells. In one embodiment, the cells are vertebrate
cells. In a further embodiment, the vertebrate cells
are mammalian cells. In a still further embodiment,
15 the mammalian cells are human cells.

The invention provides a chemical compound identified
by any of the methods described herein.

20 The invention provides a pharmaceutical composition
comprising (a) an amount of a chemical compound
identified using any of the methods described herein,
or a novel structural and functional homolog or
analog thereof, capable of passing through a cell
25 membrane and effective to increase intracellular
cyclin-dependent kinase inhibitor p27 activity and
(b) a pharmaceutically acceptable carrier capable of
passing through the cell membrane.

30 The invention provides a pharmaceutical composition
comprising an amount of a chemical compound
identified using any of the methods described herein

The invention provides a method for preparing a composition which comprises admixing a carrier and a pharmaceutically effective amount of a chemical compound identified by any of the methods described herein or a novel structural and functional analog or homolog thereof.

The invention provides a method for making a composition of matter which inhibits cellular migration which comprises identifying a chemical compound using any of the methods described herein, and then synthesizing the chemical compound or a novel structural and functional analog or homolog thereof.

The invention provides a method of treating a subject with a cardiovascular disease which comprises administering to the subject a therapeutically effective amount of a chemical compound identified by any of the methods described herein, or a novel structural and functional analog or homolog thereof.

In different embodiments, the cardiovascular disease is atherosclerosis, arteriopathy after heart transplantation, or restenosis after angioplasty or coronary stent placement.

The invention provides a method of inhibiting tumor metastasis in a subject which comprises administering to the subject a therapeutically effective amount of a chemical compound identified by any of the methods

described herein, or a novel structural and functional analog or homolog thereof.

5 The invention provides a use of a chemical compound identified by any of the methods described herein for the preparation of a pharmaceutical composition for treating an abnormality, wherein the abnormality is alleviated by inhibiting cellular migration. In different embodiments, the abnormality is a
10 cardiovascular disease or a tumor metastasis. In different embodiments, the cardiovascular disease is atherosclerosis, arteriopathy after heart transplantation, or restenosis after angioplasty or coronary stent placement.

15 In the subject invention, a "pharmaceutically effective amount" is any amount of a compound which, when administered to a subject suffering from a disease against which the compound is effective,
20 causes reduction, remission, or regression of the disease. Furthermore, as used herein, the phrase "pharmaceutically acceptable carrier" means any of the standard pharmaceutically acceptable carriers. Examples include, but are not limited to, phosphate
25 buffered saline, physiological saline, water, and emulsions, such as oil/water emulsions.

A "structural and functional analog" of a chemical compound has a structure similar to that of the
30 compound but differing from it in respect to a certain component or components. A "structural and functional homolog" of a chemical compound is one of a series of compounds each of which is formed from

the one before it by the addition of a constant element. The term "analog" is broader than and encompasses the term "homolog".

5 This invention will be better understood from the
Experimental Details which follow. However, one
skilled in the art will readily appreciate that the
specific methods and results discussed are merely
illustrative of the invention as described more fully
10 in the claims which follow thereafter.

Approved for Release
by NSA on 08-25-2014 pursuant to E.O. 13526

Experimental Details

Materials And Methods

5 Reagents: Dulbecco Modified Eagle Medium (DMEM) and
trypsin were obtained from GIBCO (Grand Island, NY),
recombinant bFGF was obtained from Biosource
International (Camarillo, CA), and paclitaxel was
obtained from Sigma (St. Louis, MO). Rapamycin was a
10 gift from Dr. Suren Sehgal (Wyeth-Ayerst Laboratories,
Princeton, NJ).

Expression of C3 exoenzyme: C3 exoenzyme was prepared
as previously described (Dillon and Feig, 1995). The
15 Glutathione S Transferase (GST)-C3 exoenzyme cDNA
(gift of Dr. Judy Meinkoth, University of
Pennsylvania) was transformed into competent BL21.
Protein expression was induced with 200 μ M
isopropylthiogalactoside (IPTG) at 32°C for 3 hours.
20 Lysates were prepared and incubated with GST-sepharose
beads for 1 hour at 4°C. The beads were washed and
incubated overnight at 4°C with 3 units/ml thrombin
(for cleavage of the C3 exoenzyme from the GST fusion
protein), which was removed by incubating the
25 supernatant with antithrombin-sepharose beads for 1
hour at 4°C. The supernatant was concentrated with a
Centricon-10 (Amicon Inc, Beverly, Mass). Protein
concentration was determined by Bradford assay and the
supernatant was aliquoted and frozen in liquid
30 nitrogen. The samples were run on SDS-PAGE and
stained with Coomassie to confirm correct expression
of the GST fusion protein and cleavage/purification of
C3 exoenzyme before use (Seasholtz et al., 1999).

Cell Culture: The murine aortic SMCs were obtained from the explant migration experiments described below, and were subcultured in DMEM containing 20% fetal bovine serum (FBS) at 37°C in a humidified 95% air-5% CO₂ atmosphere (Kobayashi et al., 1993). The growth medium was changed every other day until 80% confluence was reached. The cells used for experiments were from passages #3-6. Verification of SMC phenotype was determined by positive fluorescent staining for α -actin and negative staining for Factor VIII antigen. Cell viability was 95% or greater as determined by trypan blue exclusion at the conclusion of each experiment.

SMC Adhesion Assay: The adhesion assay was performed as previously described (Wang et al., 1997). Murine SMCs were treated with rapamycin or vehicle for 48 hours. SMCs (5×10^5 /ml in DMEM supplemented with 0.2% bovine serum albumin (BSA)) were loaded onto 12-well plates pre-coated with laminin or fibronectin. After 3 hours, the media containing nonadherent cells were removed, and cell numbers were determined by triplicate counts using a Coulter Counter (Model Z1, Coulter Electronics, Beds, England).

SMC migration assay: Migration was measured using a 48 well modified Boyden chamber housing a polycarbonate filter with 8 μ m pores as described previously (Bornfeldt et al., 1994; Poon et al., 1996). Each membrane was coated with 0.1 μ g/ml of collagen in 0.2 M acetic acid for 24 hours before each assay. For each assay, 50 ng/ml of bFGF in DMEM

was loaded in quadruplicate wells in the bottom chamber. BSA (0.2% in DMEM without bFGF) was used as a negative control. Rapamycin, FK506 or C3 exoenzyme was directly added to the growth medium for either 48 hours (rapamycin and FK506) or 16 hours (C3 exoenzyme) before the cells were trypsinized, and counted with a hemacytometer. An equal number of cells ($2 \times 10^5/\text{ml}$) in 50 μl was loaded to the top chamber of each well. After 6 hours, non-migrating cells were scraped from the upper surface of the filter. Cells on the lower surface were fixed with methanol and stained with Giemsa stain (Fisher Scientific, NY). The number of SMC on the lower surface of the filter was determined by counting four high power (X200) fields of constant area per well. Values are expressed as the percentage of cells migrating in response to bFGF after subtraction of the negative control (DMEM + BSA). Experiments were performed at least twice using quadruplicate wells.

Aortic SMC explant migration: Wild type C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, Maine). The p27(+/-) and p27 (-/-) knockout mice were kindly provided by Dr. Andrew Koff of Memorial Sloan-Kettering Cancer Institute (Kiyokawa et al., 1996). The mice received one of three different treatment protocols (9mg/kg/day for 7 days, 4 mg/kg/day for 5 days, or 2 mg/kg/day for 2 days) of rapamycin via intraperitoneal (IP) injection. The control group was treated with vehicle alone (0.2% sodium CMC, polysorbate 0.25%; Sigma, St. Louis, MO). At the conclusion of the treatment protocol, the mice were euthanized with 100 mg/kg of pentobarbital, the

aortas excised and the adventitia and surrounding connective tissue were removed. The aortas were then opened by a longitudinal cut and the intima, as well as a thin portion of the subjacent media, were removed. The media were divided into 2 mm X 2 mm pieces and placed in 6 well tissue culture plates (35mm, 22.6mm diameter, Costar, Cambridge, MA) containing DMEM with 20% FBS. The culture media was changed every other day. The migration of SMC out of the explant was observed under the microscope daily following explant. The total number of cells explanted was determined for each animal's explants on a daily basis. The results in Figure 5 are presented as the mean percentage (\pm SD) of inhibition of migration (by rapamycin or taxol) as compared to control (untreated) for at least 4 animals from each group. The SMC phenotype was confirmed as previously described (Spector et al., 1997).

Immunoblots: Immunoblots were prepared using procedures previously described in Luo et al. (1996). SMC growing in log phase or treated with rapamycin (100 nM for 48 hours) were washed twice with ice cold phosphate buffered saline (PBS) and lysates prepared using a modified RIPA buffer as previously described (Poon et al., 1996). Lysates were clarified by centrifugation for 20 minutes at 14,000 rpm at 4°C. Protein concentrations were determined by Bradford assay with BSA as a standard (Bradford, 1976). Protein extracts (30 μ g) were size-fractionated on SDS-12% polyacrylamide gels and transferred to nitrocellulose. Filters were blocked with PBS-0.1% Tween 20 and 5% dry milk for 1 hour at room

temperature, followed by incubation with a mouse monoclonal p27^{kpl} antibody (F8 antibody, Santa Cruz Biotechnology Inc, Santa Cruz, CA) for 2 hours. Filters were washed with PBS-0.1% Tween 20 and then incubated with a secondary antibody conjugated to peroxidase for 1 hour. Filters were washed with PBS-0.1% Tween 20; signals were detected using chemiluminescence detection system (ECL) followed by exposure to Kodak XAR film.

Statistics: Data are presented as the mean \pm standard deviation (SD) of the independent experiments. Statistical significance was determined by one way analysis of variance (ANOVA) and Fisher's PLSD test (StatView 4.01; Brain Power, Inc., Calabasas, CA). A paired t test (StatView 4.01) was used to analyze all data. A p value of < 0.05 was considered statistically significant.

Results

The inhibitory effects of rapamycin on the migration of SMCs isolated from wild type and p27 (-/-) knockout mice were determined. In wild type murine SMC, rapamycin treatment for 48 hours demonstrated a significant inhibitory effect on bFGF-induced SMC migration (Figure 1A, open bars). The inhibition was concentration dependent between 1 nM and 100 nM, with an IC₅₀ of ~2 nM. In contrast, no significant inhibition of migration by rapamycin (1 nM to 10 nM) was observed in the p27 (-/-) SMC (Figure 1B, open bars). At higher concentrations (100 nM), an approximately 35% inhibition was observed; the IC₅₀ in

p27 (-/-) cells was ~200 nM, representing a 100 fold increased IC₅₀ as compared to wild type SMC. Addition of rapamycin to either the upper or lower chambers immediately prior to incubation had no effect on SMC migration. FK506, an agent that binds to the same cytosolic receptor (FKBP12) as rapamycin, had no effect on murine SMC migration (Figure 1A and 1B, blackened bars). The inhibition of migration of wild type murine SMC by rapamycin (10 nM) was competitively inhibited by a 100-fold molar excess of FK506 (Figure 1C). The rapamycin-induced inhibition of migration (100 nM) in the p27 (-/-) SMC was also competitively inhibited by a 20 fold molar excess of FK506 (Figure 1D). These data indicate that the inhibition of migration was mediated through rapamycin's binding to FKBP12. Treatment of wild type murine SMC with rapamycin (100 nM for 48 hours) caused a significant increase in p27^{kip1} protein levels (Figure 1A, inset); in contrast, no p27^{kip1} was detected in p27 (-/-) SMC (Figure 1B, inset). Although rapamycin inhibits SMC proliferation, the differences in migration do not reflect proliferation as equal numbers of cells were loaded into the Boyden chamber. To confirm this, the numbers of cells in the upper and lower chambers after the 6 hour incubation were equal in the untreated and treated wild type and p27 (-/-) SMC. In addition, no differences in cell viability were noted between untreated and rapamycin treated SMC obtained from wild type and p27 (-/-) animals. No morphologic differences were observed between untreated and rapamycin (100 nM for 48 hours) treated SMC isolated from wild type mice and p27 (-/-) mice.

Since migration is dependent upon the adhesion of the SMC to the Boyden chamber membrane, adhesion assays were performed using fibronectin and laminin-coated plates. SMC obtained from p27 (-/-) animals demonstrated no differences in adhesion as compared to SMC obtained from wild type animals on both fibronectin and laminin coated plates. Furthermore, rapamycin treatment (100 nM for 48 hours) did not affect cell adhesion in either wild type or p27 (-/-) SMC (Figure 2).

To assess the *in vivo* effects of rapamycin on SMC migration in the p27 (-/-) animals, the ability of SMC to migrate out of the murine aortic explants and establish cell cultures was examined. Rapamycin was not added to the culture medium after the aortas were explanted. Explant migration of aortic SMC was performed using wild type C57BL/6, p27 (+/-), or p27 (-/-) mice. SMC from wild type, p27 (+/-) and p27 (-/-) migrated out of the aortic explant by day #2. In animals treated with rapamycin (4 mg/kg/day for 5 days), ~85% inhibition of migration as compared to untreated animals was observed in the wild type and p27 (+/-) groups ($p < 0.05$). In contrast, no rapamycin-mediated inhibition of migration was observed in p27 (-/-) group ($p < 0.05$, Figure 3A), indicating that p27^{kip1} plays a critical role in the rapamycin-mediated inhibition of SMC migration. At higher doses (9 mg/kg/day for 7 days), equivalent levels of rapamycin-mediated inhibition of migration were observed in wild type, p27 (+/-) and p27 (-/-) cells (Figure 3B). At lower doses (2 mg/kg/day for 2

days), no rapamycin-mediated inhibition of migration was observed. These results are consistent with the findings obtained in the modified Boyden chamber for p27 (-/-) cells and suggests the presence of both p27^{kip1}-dependent and p27^{kip1}-independent pathways mediating rapamycin's SMC anti-migratory actions. In order to demonstrate that agents that did not perturb the p27^{kip1} pathway could inhibit migration in p27(-/-) animals, wild type and p27 (-/-) animals were treated with taxol (20 mg/kg/day for 7 days) (Sollott et al., 1995). No differences in taxol-mediated inhibition were observed in the two groups (Figure 3C).

Recent data suggests that the Ras/RhoA mitogenic pathway regulates the destruction of p27^{kip1}. C3 exoenzyme, which adenosine diphosphate (ADP)-ribosylates and inactivates RhoA, inhibited PDGF-induced p27^{kip1} degradation. These findings suggest that activation of RhoA by mitogens is necessary for degradation of p27^{kip1} (Weber et al., 1997). In addition, thrombin-induced vascular SMC DNA synthesis and migration were inhibited by C3 exoenzyme (Seasholtz et al., 1999). We sought to determine whether this inhibition of migration was mediated, in part, by regulating p27^{kip1} levels. SMC from wild type and p27 (-/-) animals were exposed to either 2 µg/ml or 20 µg/ml C3 exoenzyme for 16 hours, trypsinized and loaded into the upper chamber of the Boyden chamber. C3 exoenzyme significantly inhibited bFGF-mediated SMC migration in wild type cells (Figure 4, open bars). SMC from p27 (-/-) animals demonstrated a 25% relative resistance to C3 exoenzyme (Figure 4,

blackened bars). SMC that were acutely exposed to C3
exoenzyme demonstrated no inhibition of migration.
These results implicate p27^{kip1} as a regulator, in
part, of both rapamycin and C3 exoenzyme-mediated
5 inhibition of SMC migration.

Discussion

Rapamycin has been shown previously to inhibit rat,
10 porcine, and human SMC migration (Poon et al., 1996).
In addition, rapamycin reduces intimal thickening by
50% after coronary angioplasty in the porcine model
(Gallo et al., 1999). The rapamycin anti-restenotic
effect is characterized by an inhibition of the SMC
15 response to coronary injury with a concomitant
decrease in retinoblastoma protein (pRb)
phosphorylation as well as an increase in p27^{kip1}
levels, thereby resulting in cell-cycle arrest (Gallo
et al., 1999; Marx et al., 1995). The cyclin-
20 dependent kinase inhibitor (CDKI) p27^{kip1} inhibits the
regulatory activities of cyclin/CDK complexes
including cyclinE/CDK2 by directly binding to them
and, in turn, blocking the phosphorylation of
retinoblastoma protein (pRb) (Kato et al., 1994;
25 Nourse et al., 1994). Thus, p27^{kip1} is a regulator of
cell proliferation; reduction of p27^{kip1} protein levels
during the late G₁ phase is required for cyclin/CDK
complex activation and cell cycle progression in
certain cell lines. The CDKI p27^{kip1} is present at
30 high levels in quiescent cells and upon mitogenic
stimulation is downregulated (Kato et al., 1994;
Nourse et al., 1994). Down-regulation of p27^{kip1} by

mitogens can be blocked by the immunosuppressant rapamycin (Nourse et al., 1994).

5 The function of p27^{Kip1} is clinically relevant because
of the connections that have been made between the
down-regulation and enhanced degradation of p27^{Kip1} in
colorectal, stomach, breast, and small-cell lung
cancers (Steeg and Abrams, 1997). Furthermore, the
regulation of the CDKI p27^{Kip1} plays a critical role in
10 the regulation of SMC proliferation in vivo.
Decreased levels of p27^{Kip1} in the vessel wall has been
associated with increased neointimal response after
percutaneous transluminal angioplasty (PTCA) (Braun-
Dullaes and al., 1997; Tanner et al., 1998).
15 Angiotensin II stimulation of quiescent vascular SMC
in which p27^{Kip1} levels are high results in SMC
hypertrophy but induces SMC hyperplasia when levels
of p27^{Kip1} are low as occurs in the presence of
mitogens (Braun-Dullaes et al., 1999). The findings
20 disclosed in the present application suggest that
agents that increase p27^{Kip1} levels in vivo may have
both an anti-proliferative and anti-migratory effect.

25 Although the regulation of p27^{Kip1} can occur at the
mRNA level (Hengst and Reed, 1996), most studies have
supported the concept that p27^{Kip1} is regulated post-
transcriptionally and involves ubiquitin (Ub)-
proteasome dependent degradation (Pagano et al.,
1995). Targeting of p27^{Kip1} for ubiquitin is believed
30 to involve phosphorylation of p27^{Kip1} by cyclin E-cdk2
complex (Sheaff et al., 1997; Vlach et al., 1997).
Recently, a ubiquitin-proteasome independent pathway
has been described that involves proteolytic

processing that rapidly clips off the cyclin-binding domain. This ubiquitin independent processing is ATP-dependent and sensitive to proteasome-specific and chymotrypsin inhibitors (Shirane et al., 1999).

5

In addition, p27^{kip1} levels have been shown to be regulated by the Ras/RhoA mitogenic pathway. Overexpression of a dominant negative Ras or RhoA inhibited the platelet derived growth factor (PDGF) induced degradation of p27^{kip1}. C3 exoenzyme, which ADP-ribosylates and inactivates RhoA, inhibited PDGF-induced p27^{kip1} degradation (Hirai et al., 1997; Weber et al., 1997) and inhibited thrombin-mediated vascular SMC proliferation and migration (Seasholtz et al., 1999). In Swiss 3T3 fibroblasts, it has been shown that Rho can be activated by extracellular ligands (lysophosphatidic acid) and that Rho activation can lead to the assembly of contractile actin-myosin filaments and focal adhesion complexes (Hall, 1998). Rac, a member of the Rho subfamily, has been shown to induce actin-rich surface protrusions (filopodia); Rac can activate Rho (although in fibroblasts this interaction is weak and delayed) (Hall, 1998). Generation of phosphatidylinositol-3,4,5-trisphosphate (PIP3) by PI 3-kinase activity is essential for receptor-mediated activation by Rac in mammalian cells and a PI3 kinase homolog, TOR2 (target of rapamycin 2) controls RhoA activation in *Saccharomyces cerevisiae* (Hall, 1998; Schmidt et al., 1997). These observations suggests that the Rho GTPase family is one of the key regulatory molecules that link surface receptors to the organization of the actin cytoskeleton.

10

15

20

25

30

Rapamycin has not been shown to interact with the Rho GTPase family, although it is interesting that inhibition of both Rho (Hirai et al., 1997; Weber et al., 1997) and mTOR (Brown et al., 1994; Nourse et al., 1994; Sabatini et al., 1994) are both associated with increased levels of the CDKI, p27^{kip1}.

The extracellular matrix (ECM) plays an essential role in the regulation of cell proliferation. Human capillary endothelial cells that were prevented from spreading (either mechanically or pharmacologically with cytochalasin or actomyosin) exhibited normal activation of mitogen-activated kinases, but failed to progress through G1 phase (Huang et al., 1998). This shape dependent block in the cell cycle was correlated with a failure to down-regulate p27^{kip1}, up-regulate cyclin D1 and phosphorylate pRb (Huang et al., 1998). Therefore, the accumulation of p27^{kip1} in cells prevented from spreading suggests that p27^{kip1} could play a role in the shape-dependent cell cycle arrest produced by cell rounding. Signaling pathway components that could be responsible for transducing the accumulation of p27^{kip1} include Rho, which is involved in integrin-mediated changes in the cytoskeleton tension and shape, and the integrin-linked kinase, which has been shown to reduce the inhibitory actions of p27^{kip1} and to promote anchorage-independent growth (Chrzanowska-Wodnicka and Burridge, 1996; Hotchin and Hall, 1995; Huang et al., 1998; Radeva et al., 1997).

The p21 CDKI (Cip1) has been shown to inhibit SMC migration *in vitro* (Fukui et al., 1997; Witzenbichler

et al., 1999). The spreading and attachment of the p21^{Cip1} transfected rabbit aortic SMC to extracellular matrices (ECM) were inhibited compared to that of control vector-transfected cells. Cip1 transfected SMC maintained a round conformation on fibronectin. Moreover, p21^{Cip1} transfected SMC demonstrated significantly reduced PDGF-BB mediated migration in a modified Boyden chamber (with fibronectin coated membranes). Therefore, p21^{Cip1} probably acts as an adhesion inhibitor, since it prevents the assembly of actin filaments and the translocation of adhesion molecules (Fukui et al., 1997). Interestingly, our study indicates that induction of p27^{Kip1} with rapamycin did not affect adhesion to collagen of either wild type or p27 (-/-) cells.

The homeobox transcription factor Gax is expressed in quiescent vascular SMC and is down-regulated during SMC proliferation and vascular injury (Witzenbichler et al., 1999). Gax up-regulates p21^{Cip1} and inhibits vascular SMC proliferation and migration (Witzenbichler et al., 1999). p21^{Cip1} mediates the growth inhibitory actions of Gax; overexpression of Gax does not have anti-proliferative or anti-migratory effects in cells derived from p21 (-/-) mice (Smith et al., 1997; Witzenbichler et al., 1999). Gax was unable to inhibit the migration of fibroblasts which lacked p21^{Cip1} (Witzenbichler et al., 1999). Transfection of a Gax cDNA inhibited PDGF-, bFGF-, and hepatocyte growth factor-induced vascular SMC migration (Witzenbichler et al., 1999). Cell cycle arrest by either p16 or p21 is essential for Gax-induced inhibition of migration. Interestingly,

overexpression of Gax cDNA, which increases p21^{cip1},
had no effect on the adhesion of cells to collagen
and vitronectin coated plates. Therefore, in
contrast to the fibronectin adhesion defect shown in
5 cells transfected with p21^{cip1}, cells transfected with
Gax cDNA demonstrated no collagen/vitronectin
adhesion defect. However, the studies reported
conflicting information regarding the effects of
overexpression of p21^{cip1} on SMC migration; p21^{cip1}
10 transfection of rabbit vascular SMC inhibited
migration in a fibronectin coated Boyden chamber
(Fukui et al., 1997), whereas p21^{cip1} transfection in
rat vascular SMC had no effect in a
collagen/vitronectin Boyden chamber (Witzenbichler et
15 al., 1999).

In conclusion, rapamycin and C3 exoenzyme inhibit
smooth muscle cell migration through p27^{kip1}-dependent
and independent pathways (Figure 5). This intriguing
20 finding implicates p27^{kip1} in the signaling pathway(s)
that regulate both SMC proliferation and migration.
Technologies (e.g., pharmacologic, recombinant and/or
gene therapy) aimed at increasing p27^{kip1} are expected
to have dramatic effects on the amelioration of
25 restenosis after angioplasty or stent placement, or
on accelerated arteriopathy after cardiac
transplantation, as well as in cancer therapy where
cellular migration is a key element in tumor
metastasis.

References

- Bornfeldt, K. E., Raines, E. W., Nakano, T., Graves, L. M., Krebs, E. G., and Ross, R. (1994). Insulin-like growth factor-I and platelet-derived growth factor-BB induce directed migration of human arterial smooth muscle cells via signaling pathways that are distinct from those of proliferation. *J Clin Invest* 93, 1266-1274.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Anal Biochem* 72, 248-254.
- Braun-Dullaeus, R. C., and al., e. (1997). Loss of p27kip1 and induction of Cdk1 in the rat carotid artery following balloon catheter injury. In vivo and in vitro influence of rapamycin. *FASEB J* 11, A153 (abstract).
- Braun-Dullaeus, R. C., Mann, M. J., Ziegler, A., von der Leyen, H. E., and Dzau, V. J. (1999). A novel role for the cyclin-dependent kinase inhibitor p27kip1 in angiotension II-stimulated vascular smooth muscle cell hypertrophy. *J. Clin. Invest.* 104, 815-823.
- Brown, E., Albers, T., Shin, T., Ichikawa, K., Keith, C., Lane, W., and Schreiber, S. (1994). A mammalian protein targeted by G1-arresting rapamycin complex. *Nature (London)* 369, 756-758.

Cao, W., Mohacsi, P., Shorthouse, R., Pratt, R., and
Morris, R. (1995). Effects of rapamycin on growth
factor-stimulated vascular smooth muscle cell DNA
synthesis: inhibition of basic fibroblast growth
5 factor and platelet-derived growth factor action and
antagonism of rapamycin by FK506. Transplantation 59,
390-395.

Chrzanowska-Wodnicka, M., and Burridge, K. (1996).
10 Rho-stimulated contractility drives the formation of
stress fibers and focal adhesions. J. Cell Biol. 133,
1403-1415.

Clowes, A., and Schwartz, S. M. (1985). Significance
15 of quiescent smooth muscle migration in the injured
rat carotid artery. Circ Res. 56, 139-45.

Dillon, S. T., and Feig, L. A. (1995). Purification
and assay of recombinant C3 transferase. Methods in
20 Enzymology 256, 174-184.

Ferns, G. A. A., Raines, E. W., Sprugel, H., Motani,
A. S., Reidy, M. A., and Ross, R. (1991). Inhibition
of neointimal smooth muscle accumulation after
25 angioplasty by an antibody to PDGF. Science 253,
1129-1132.

Fukui, R., Shibata, N., Kohbayashi, E., Amakawa, M.,
Furutama, D., Hoshiga, M., Negoro, N., Nakakouji, T.,
30 Ii, M., Ishihara, T., and Ohsawa, N. (1997).
Inhibition of smooth muscle cell migration by the p21
cyclin-dependent kinase inhibitor (Cip1).
Atherosclerosis 132, 53-59.

- Gallo, R., Padurean, A., Jayaraman, T., Marx, S. O.,
Roque, M., Adelman, S., Chesebro, J., Fallon, J.,
Fuster, V., Marks, A. R., and Badimon, J. J. (1999).
5 Inhibition of intimal thickening after balloon
angioplasty in porcine coronary arteries by targeting
regulators of the cell cycle. *Circulation* 99, 2164-
2170.
- 10 Gregory, C., Huie, P., Billingham, M., and Morris, R.
(1993). Rapamycin inhibits arterial intimal
thickening caused by both alloimmune and mechanical
injury. *Transplantation* 55, 1409-1418.
- 15 Grotendorst, G. R., Seppa, H. E. J., Kleinman, H. K.,
and Martin, G. R. (1981). Attachment of smooth muscle
cells to collagen and their migration toward platelet
derived growth factor. *Proc Natl Acad Sci USA* 78,
3669-3672.
- 20 Hall, A. (1998). Rho GTPases and the actin
cytoskeleton. *Science* 279, 509-514.
- Hengst, L., and Reed, S. I. (1996). Translational
25 control of p27^{kip1} accumulation during the cell
cycle. *Science* 271, 1861-1864.
- Higaki, M., Sakaue, H., Ogawa, W., Kasuga, M., and
Shimokado, K. (1996). Phosphatidylinositol 3-kinase-
30 independent signal transduction pathway for platelet-
derived growth factor-induced chemotaxis. *J. Biol.*
Chem. 271, 29342-29346.

- Hirai, A., Nakamura, S., Noguchi, Y., Yasuda, T., Kitagawa, M., Tatsuno, I., Oeda, T., Tahara, K., Terano, T., Narumiya, S., Kohn, L. D., and Saito, Y. (1997). Geranylgeranylated rho small GTPase(s) are essential for the degradation of p27kip1 and facilitate the progression from G1 to S phase in growth-stimulated rat FRTL-5 cells. *J. Biol. Chem.* 272, 13-16.
- Hotchin, N. A., and Hall, A. (1995). The assembly of integrin adhesion complexes requires both extracellular matrix and intracellular rho/rac GTPases. *J. Cell. Biol.* 131, 1857-65.
- Huang, S., Chen, C. S., and Ingber, D. E. (1998). Control of cyclin D1, p27kip1 and cell cycle progression in human capillary endothelial cells by cell shape and cytoskeletal tension. *Mol. Biol. Cell* 9, 3179-3193.
- Ihnatowycz, I. O., Winocour, P. D., and Moore, S. (1981). A platelet-derived factor chemotactic for rabbit smooth muscle cells in culture. *Artery* 9, 316-317.
- Jawien, A., Bowen-Pope, D. F., Lindner, V., Schwartz, S. M., and Clowes, A. W. (1992). Platelet-derived growth factor promotes smooth muscle migration and intimal thickening in a rat model of balloon angioplasty. *J Clin Invest.* 89, 507-511.
- Kato, J. M., Matsuoka, M., Polyak, K., Massague, J., and Sherr, C. J. (1994). Cyclic AMP-induced G1 phase

arrest mediated by an inhibitor (p27^{kip1}) of cyclin-dependent kinase-4 activation. Cell 79, 487-496.

5 Kiyokawa, H., Kineman, R. D., Manova-Todorova, K. O.,
Soares, V. C., Hoffman, E. S., Ono, M., Khanam, D.,
Hayday, A. C., Frohman, L. A., and Koff, A. (1996).
Enhanced growth of mice lacking the cyclin-dependent
kinase inhibitor function of p27^{kip1}. Cell 85, 721-
732.

10 Kobayashi, S., Mimura, Y., Naitoh, T., Kimura, I.,
and Kimura, M. (1993). Chemical structure-activity of
cnidium rhizome-derived phthalides for the competence
inhibition of proliferation in primary culture of
15 mouse aorta smooth muscle cells. Japan J. Pharmacol
63, 353-359.

Luo, Y., Marx, S. O., Kiyokawa, H., Koff, A.,
Massague, J., and Marks, A. R. (1996). Rapamycin
20 resistance tied to defective regulation of p27^{kip1}.
Mol. Cell. Biol. 16, 6744-6751.

Marx, S. O., Jayaraman, T., Go, L. O., and Marks, A.
R. (1995). Rapamycin-FKBP inhibits cell cycle
25 regulators of proliferation in vascular smooth muscle
cells. Circ Res 76, 412-417.

Marx, S. O., and Marks, A. R. (1999). Cell cycle
progression and proliferation despite 4BP-1
30 dephosphorylation. Mol Cell Biol. 19, 6041-6047.

Nourse, J., Firpo, E., Flanagan, W. M., Coats, S., Polyak, K., Lee, M., Massague, J., Crabtree, G., and Roberts, J. M. (1994). Interleukin-2-mediated elimination of the p27^{kip1} cyclin-dependent kinase inhibitor prevented by rapamycin. *Nature*(London) 372, 570-573.

Pagano, M., Tam, S. W., Theodoras, A. M., Beer-Romero, P., G., D. S., Chau, V., Yew, P. R., Draetta, G. F., and Rolfe, M. (1995). Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. *Science* 269, 682-685.

Pickering, J. G., Uniyal, S., Ford, C. M., Chau, T., Laurin, M. A., Chow, L. H., Ellis, C. G., Fish, J., and Chan, B. (1997). Fibroblast growth factor-2 potentiates vascular smooth muscle cell migration to platelet-derived growth factor: upregulation of $\alpha 2 \beta 1$ integrin and disassembly of actin filaments. *Circ Res.* 80, 627-37.

Poon, M., Marx, S. O., Gallo, R., Badimon, J. J., Taubman, M. B., and Marks, A. R. (1996). Rapamycin inhibits vascular smooth muscle cell migration. *J. Clin. Invest.* 98, 2277-2283.

Poston, R. S., Billingham, M., Hoyt, E. G., Pollard, J., Shorthouse, R., Morris, R. E., and Robbins, R. C. (1999). Rapamycin reverses chronic graft vascular disease in a novel cardiac allograft model. *Circulation* 100, 67-74.

Radeva, G., Petrocelli, T., Behrend, E., Leung-Hagesteijn, C., Filmus, J., Slingerland, J., and Dedhar, S. (1997). Overexpression of the integrin-linked kinase promotes anchorage-independent cell cycle progression. *J. Biol. Chem.* 272, 13937-13944.

Sabatini, D. M., Erdjument-Bromage, H., Lui, M., Tempst, P., and Snyder, S. H. (1994). RAFT1: A mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. *Cell* 78, 35-43.

Schmidt, A., Bickle, M., Beck, T., and Hall, M. N. (1997). The yeast phosphatidylinositol kinase homolog TOR2 activates RHO1 and RHO2 via the exchange factor ROM2. *Cell* 88, 531-542.

Schwartz, S. M. (1997). Smooth muscle migration in atherosclerosis and restenosis. *J. Clin. Invest.* 100, S87-98.

Seasholtz, T. M., Majumdar, M., Kaplan, D. D., and Brown, J. H. (1999). Rho and Rho kinase mediate thrombin-stimulated vascular smooth muscle cell DNA synthesis and migration. *Circ Res* 84, 1186-1193.

Sheaff, R. J., Groudine, M., Gordon, M., Roberts, J. M., and Clurman, B. E. (1997). Cyclin E-CDK2 is a regulator of p27^{kip1}. *Genes Dev.* 11, 1464-1478.

Shirane, M., Harumiya, Y., Ishida, N., Hirai, A., Miyamoto, C., Hatakeyama, S., Nakayama, K., and Kitagawa, M. (1999). Down-regulation of p27^{kip1} by

two mechanisms, ubiquitin-mediated degradation and proteolytic processing. *J Biol Chem* 274, 13886-13893.

- 5 Smith, R. C., Branellec, D., Gorski, D. H., Guo, K., Perlman, H., Dedieu, J. F., Pastore, C., Mahfoudi, A., Deneffe, P., Isner, J. M., and Walsh, K. (1997). p21cip1-mediated inhibition of cell proliferation by overexpression of the *gax* homeodomain gene. *Genes Dev.* 11, 1674-89.
- 10 Sollott, S. J., Cheng, L., Pauly, R. R., Jenkins, G. M., Monticone, R. E., Kuzuya, M., Froehlich, J. P., Crow, M. T., Laketta, E. G., Rowinsky, E. K., and Kinsella, J. L. (1995). Taxol inhibits neointimal smooth muscle cell accumulation after angioplasty in the rat. *J. Clin. Invest.* 95, 1869-1876.
- 15 Sousa, J. E., Costa, M. A., Abizaid, A., Abizaid, A. S., Feres, F., Pinto, I.M.F. et al. (2000) Lack of neointimal proliferation after implantation of sirolimus-coated stents in human coronary arteries. A quantitative coronary angiography and three-dimensional intravascular ultrasound study. *Circulation* 102, r54-r57.
- 20 Spector, D. L., Goldman, R. D., and Leinwand, L. A. (1997). *Cells: a laboratory manual*. (New York: Cold Spring Harbor Laboratory Press).
- 25 Steeg, P. S., and Abrams, J. S. (1997). Cancer prognostics: Past, present and p27. *Nature Med.* 3, 152-154.
- 30

Tanner, F. C., Yang, Z. Y., Duckers, E., Gordon, D., Nabel, G. J., and Nabel, E. G. (1998). Expression of cyclin-dependent kinase inhibitors in vascular disease. *Circ Res* 82, 396-403.

5

Vlach, J., Hennecke, S., and Amati, B. (1997). Phosphorylation-dependent degradation of the cyclin-dependent kinase inhibitor p27^{kip1}. *EMBO J.* 16, 5334-5344.

10

Wang, W., Chen, H. J., Warshofsky, M., Schwartz, A., C.A., S., and L.E., R. (1997). Effects of S-dC28 on vascular smooth muscle cell adhesion and plasminogen activator production. *Antisense & Nucleic Acid Drug Development* 7, 101-107.

15

Weber, J. D., Hu, W., Jefcoat, S. C., Raben, D. M., and Baldassare, J. J. (1997). Ras-stimulated extracellular signal-related kinase 1 and RhoA activities coordinate platelet-derived growth factor-induced G1 progression through the independent regulation of cyclin D1 and p27^{kip1}. *J Biol Chem* 272, 32966-32971.

20

Witzenbichler, B., Kureishi, Y., Luo, Z., Le Roux, A., Branellec, D., and Walsh, K. (1999). Regulation of smooth muscle cell migration and integrin expression by the Gax transcription factor. *J. Clin. Invest.* 104, 1469-1480.

25

30